AN EVALUATION OF AN IN SITU FLUOROMETER FOR THE ESTIMATION OF CHLOROPHYLL A

by John Marra and Christopher Langdon

TECHNICAL REPORT

LDEO-93-1

Department of the Navy
Office of Naval Research
Contract N00014-C-0132

Lamont-Doherty Earth Observatory
of Columbia University
Palisades, New York 10964

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ABSTRACT

In situ fluorometers are evaluated in their estimation of chlorophyll a. Calibrations from at-sea and laboratory data showed linear relationships between fluorescence and chlorophyll a, as measured by in situ fluorometers with $r^2 > 0.9$. Examination of regression residuals showed an increasing error variance with the magnitude of chlorophyll on two of four cruises. The most likely source of this increasing error variance was in one case, a photoadaptation effect and in the other a population shift between the beginning and end of the cruise. Smaller variability was also found in the ratio fluorescence to chlorophyll a, traced to sample depth, and time of day, although this variability was not a consistent property of the data. Generally, there was excellent agreement between laboratory and at-sea calibrations for low levels of chlorophyll typical of oceanic environments. The laboratory calibration of these instruments was stable over time, suggesting that good estimates of chlorophyll a can be made from fluorometers placed on ocean moorings.

INTRODUCTION

In situ fluorometers are used more and more at sea (e.g., Aiken, 1981; Whitledge and Wirick, 1983; Weller et al., 1985; Marra et al. 1990) and in lakes (e.g., Heaney, 1978; Abbott et al., 1982). However, worries have been reported regarding the ability of in vivo fluorescence to estimate accurately chlorophyll a. For example, Cullen (1982) doubts that fluorescence could be linearly related to chlorophyll a given the variability of chlorophyll absorption and the variability of the fluorescence yield, concluding that fluorescence profiles should be interpreted in their own right, separate from chlorophyll a. Falkowski and Kiefer (1985) state that the

interpretation of the fluorescence signal is not simple, nor is it a linear function of chlorophyll, and echo the sources of variability mentioned in Cullen (1982). Vandevelde et al. (1988) also urge caution, partly because of the variation in fluorescence yield per unit chlorophyll.

That fluorescence could merely be an "indicator of chlorophyll" (Cullen et al., 1988) reflects much of these concerns. While no one questions that the source of the fluorescence signal is chlorophyll a, many believe that fluorescence is not a good estimator for it. These uncertainties stem from the imprecision of the conversion of the fluorescence signal to chlorophyll a, but also from the inability to discriminate the errors in the analysis of chlorophyll a from "noisiness" in the fluorescence signal. Errors of the former kind are variations in fluorescence per unit chlorophyll a which we shall designate R, following Cullen (1982), and has the units: volts (μg chlorophyll a $l^{-1})^{-1}$).

We review the calibration of in situ fluorometers for data taken during the research program Biowatt and Marine Light-Mixed Layers (ML-ML), by examining residuals and variability in R. We also include comparisons of calibrations done at-sea with those performed in the laboratory. The in situ fluorometers used in this study are all manufactured by SeaTech, Inc. (Corvallis, Oregon, 97339, U.S.A.)

MATERIALS AND METHODS

The data are from four cruises; three to the Sargasso Sea (as part of Biowatt, in 1987) and one to the Gulf of Maine. In addition, since we used these same fluorometers on the Biowatt mooring (see Dickey et al., 1990), we report on the stability of laboratory calibrations. Two types of calibrations are used here, at-sea and laboratory. At sea, the fluorometers were measured against natural populations, and measurements of chlorophyll a using a bench-top Turner fluorometer. The laboratory measurements used cultured populations whose chlorophyll was determined spectrophotometrically. Both types of calibration were ultimately referenced to a chlorophyll a standard.

At-Sea Calibration. The in situ fluorometers were mounted on the frame which carried the CTD, rosette samplers (10 l Niskin Go-Flo's), and a 25 cm-pathlength

beam transmissometer (Bartz et al. 1978). The sensor head on the fluorometer was about 0.5 m below the mid-point of the rosette sampler. The fluorometers had an excitation wavelength peak at 425 nm (200 nm FWHM) and an emission peak at 685 nm (30 nm FWHM). The fluorescence signal in these units was smoothed with a filter having a 3.0 s time constant. There are three levels of sensitivity for these fluorometers, corresponding to approximate maximum chlorophyll concentrations of 3, 10 and 30 $\mu g\ l^{-1}$, and we used the highest sensitivity setting. CTD casts were done usually every 4-6 h while on station, weather and other ship operations permitting. Samples for calibration of the in situ fluorometer were collected on each CTD cast at all depths sampled with the Go-Flo's.

The chlorophyll analysis procedure followed that described in Smith et al. (1981). Briefly, 100-500 ml of sample was filtered through a Millipore HA (pore size 0.45 μm) or Whatman GF/F filter. The filtered material was extracted for 24 h in 90% acetone and the extract's fluorescence (before and after acidification) was measured on a Turner 111 fluorometer calibrated using pure chlorophyll a.

Laboratory Calibration. For the phytoplankton culture, we used *Thalassiosira pseudonana*, a small centric diatom, in exponential phase of growth. Chlorophyll a levels in the culture were in the range of 100-300 $\mu g\ l^{-1}$. We filtered a known amount of seawater using Millipore HA filters. This seawater was placed in a black container, and the fluorometer immersed in this bath for the calibration. Immediately before beginning the calibration, an aliquot of the culture was filtered and the filter analyzed for chlorophyll a using the spectrophotometric method (Parsons et al., 1984). As a check against background fluorescence, an aliquot of the filtered seawater bath was taken and analyzed on a Turner Model 10 laboratory fluorometer using the standard chlorophyll filter set. For the calibration, known amounts of culture (i.e., known amounts of chlorophyll a, in vivo), were added to the bath, taking readings of fluorometer output after each addition.

RESULTS

Table 1 lists the duration and average euphotic zone depth for three Biowatt II cruises in 1987, and the Marine Light-Mixed Layers (ML-ML) cruise in 1990. The chlorophyll a data used for the field calibrations in Biowatt can be found in published data reports (Baker and Smith 1987a, 1987b, 1989).

OC2, OC3 and E1 all showed a subsurface fluorescence maximum and a stratified water column (Fig. 1). For OC4, there was a deep mixed layer and homogenous fluorescence throughout (Fig. 1c), although the euphotic zone was estimated to be no more than 100 m. Earlier on this cruise, there was evidence of slight near-surface stratification and increases in fluorescence, and this accounts for the wider range of chlorophyll values in the regression calibrations than indicated in the profile. Only on OC3 was there a lack of any particle maximum (as indicated by the beam attenuation coefficient (b.a.c.)) at the depth of the fluorescence maximum. The changes in b.a.c. are slight, since the beam transmissometer has about an order of magnitude less dynamic range than the in situ fluorometer.

Calibrations from the four cruises. Since we use fluorescence as an estimator for chlorophyll a, fluorescence is plotted on the x-axis and chlorophyll is plotted as the dependent variable in the calibrations from the four cruises (Fig. 2). In all cases, the regression coefficients were significant, and the $r^2 > 0.9$. (Table 2). We tested the regression for the inclusion of pheopigments, however this resulted in a lower r^2 for the regression line. Siegel and Dickey (1987), found that inclusion of pheopigments improved their regression, however, they used a different fluorometer (SeaMarTec) with different excitation and sensing characteristics.

For cruises OC2 and OC3, we obtained very good at-sea calibrations, and ones which agreed closely with the laboratory calibration (Fig. 2b,c). The fluorometer on OC2 exhibited negative voltages for low values of chlorophyll in the laboratory and at-sea (which explains the negative values for R described below). For OC3, the noise about the regression line shows a depth dependence, thus there is likely a depth dependence to R. All the samples above the regression line are from samples shallower than 100 m, while all the samples below the line are from deeper than 100 m. The laboratory calibration (and regression line, of course) bisects this sample distribution.

For OC4 there are both differences in offset and slope between the at-sea and laboratory calibrations (Fig. 2c). The high intercept on the x-axis compared to the laboratory calibration suggests that part of the fluorescence may have come either from a filterable form of particulate chlorophyll, or from a dissolved constituent exhibiting similar fluorescence properties. (See below). Cruise E1 also used the

same fluorometer as OC4, and differed between laboratory and field calibrations. This becomes especially noticeable at high values of chlorophyll, where there appears to be more fluorescent quenching in the laboratory as opposed to the field measurements.

Residual analysis. We computed the standardized residual, z (Kleinbaum and Kupper, 1978), for each sample (i), from the sample variance (S^2) and the residual (e) from the linear regression, as

$$z_i = e_i/S. \qquad (1)$$

The distribution of z_i with respect to the predicted chlorophyll a (Fig. 3.) can reveal whether or not a linear model is appropriate, or if there are any inhomogeneities in the variance. Only on OC2 (Fig.3a) do the residuals appear random. However if we ignore samples from below the eupohotic zone (i.e. <0.1 μg Chl l^{-1}), then E1 (Fig. 3d) also shows a random distribution of residuals. In contrast, OC3 and OC4 (Figs. 3b,c) the residuals increase with the magnitude of chlorophyll.

Variation of R. A further way to examine the calibrations is to normalize the fluorescence to chlorophyll a (R) to consider other sources of variation, such as the time of day, the date on which the calibration samples were collected, or sample depth. We limit ourselves to the euphotic zone for this analysis since as chlorophyll a tends to zero, R can become artificially large and skew the statistics. Also, if chlorophyll a is uniformly distributed within the euphotic zone and goes to very low values deeper, sample depth may be spuriously identified as an important variable in the statistical analysis.

The small standard errors about the regressions (Fig. 2) and the strength of the coefficient of determination (Table 2) suggest that these will be secondary sources of variation in the calibration. Multiple regression of R on these variables indicate that, for the most part, day and time of day are not significant (Table 3). The exception is OC2, where both of these appear to be a source of variability. Sample depth is important to OC2 and OC3. The sources of variation found to be highly significant to R (see Table 3) are shown in Fig. 4. The depth variation in R for OC3 shows greater scatter at shallow depths. This may be a diurnal (time of day) effect

that is manifested only at shallow depths (< 20 m), and thus does not contribute to an overall depth variability.

Stability of the laboratory calibration. Use of the in situ fluorometers as part of the sensor suite in the Biowatt Mooring Experiment (Dickey et al., 1990) meant a series of calibrations before and after each deployment. Fig. 5 shows an example of two units for which we have the most complete calibration histories. The stability of the calibrations is excellent, especially at low values of chlorophyll a, typical of the open ocean. At high values (> 1μg l^{-1}), the fluorometer apparently introduces noise into the calibration.

DISCUSSION

The sources of variability in calibrating an in situ fluorescence signal to chlorophyll a can be summarized as follows.

(1) The chlorophyll a analysis. These are procedural errors to the analysis of chlorophyll a. They include handling errors in the preparation of the acetone extract, and errors in the calibration of the at-sea fluorometer to the spectrophotometer, interferences from other chlorophylls or degradation products, and errors caused by imperfect retention by the filters of chlorophyll-containing particulates.

(2) Mismatches in sampled water volume. This is caused by the depth and time mismatches between the water volume sensed by the fluorometer (0.5 ml) and that sampled by the water-sampling bottle (liters). For practical reasons, the depth of the sensor head of the fluorometer is never at the same depth as the water-sampling bottle. Given the rapidity with which the fluorometer samples the water column for fluorescence, the time of capture of the sample in the water bottle can only be related to some averaged value of the fluorescence signal.

(3) Interpretation of the fluorescence signal. R may not be constant.
For example, photoinhibition of fluorescence is sometimes observed near the ocean surface, and may be caused by a low fluorescence yield. Alternatively, there may be

no strong change in R (and the fluorescence signal therefore interpretable in terms of chlorophyll a).

Another factor that should be considered is the distribution of values of chlorophyll a at sea. If the range of values is narrow, then the prediction of chlorophyll from fluorescence will be weakened. But establishing a wide range of values through time or over a wider spatial area, may also alter R.

(4) Interference by other chemical species. This refers to the presence of non-chlorophyll dissolved constituents which may have the same or similar fluorescence characteristics as chlorophyll a.

(5) Characteristics of the fluorometer. Variability here derives from variations in strobe output (which excites the fluorescence), the wavelength band of strobe excitation or emission spectrum, or from the intensity of the flash (Cullen et al., 1988). Also, there can be increasing variability in the signal with increasing amounts of chlorophyll.

Given the high value of r^2 in the linear regressions (Table 2), the above sources of variability are minor and do not compromise the estimate of chlorophyll from in situ fluorometry. Since there are errors in both the fluorescence and chlorophyll a values, a model II type regression (Ricker, 1973) might be required instead of the model I type used here. However, when the correlation between the two variables is high, as it is here, there is little difference between the two models (Laws and Archie, 1981).

We now examine the calibration regressions in more detail using the results from the residual analysis and the variations in R. For OC3 and OC4, the residuals clearly increase with the value of chlorophyll. This implies an increasing error variance with the magnitude of chlorophyll a (heteroscedasticity), and violates one of the assumptions of the least squares technique (that variance will be constant). We see three possible causes of the heteroscedasticity, mentioned in the above list: instrument noise (error (5) above), sampling mis-matches (error (2)) and biological variability (error (3)).

Instrument noise. An increasing error variance can be seen in the laboratory calibrations, but this occurs mostly at larger values of chlorophyll a than typically measured at sea. However, this may have caused some of the large residuals seen for E1 where chlorophyll values were in this range and much higher than on the other three cruises.

Sampling mis-matches. For the field data, variability will occur if the in situ fluorometer does not sample exactly the same depth as the Go-Flo water sampler, and this type of error would increase with the quantity of chlorophyll a, if the chlorophyll was not uniformly distributed with depth, but occurred in layers. It is possible that chlorophyll may have a high degree of variability on a depth scale of a meter or less (Derenbach et al., 1979), which because of the time constant of the sensor and lowering speed of the profiler, the fluorometer would average over, but the Go-Flo could sample. These layers might have occurred on all cruises but OC4. Since the residuals are well-behaved on OC2 and probably also on E1, this cannot be an explanation for the increasing error variance.

Biological Variability. All cruises except for OC4 exhibit some degree of changes in fluorescence relative to the beam attenuation coefficient, indicative of photoadaptation of the phytoplankton populations (Fig. 1). The data from OC3 are perhaps clearest in showing a pure fluorescence maximum, when comparing the transmissometer with the fluorescence signal. This was a likely source of the heteroscedasticity in the regression for this cruise (Fig. 3b) and suggests a non-linearity in the data in Fig. 2b. But we do not feel that recourse to a different data normalization scheme or to a weighted least-squares method is appropriate for improving the estimates. Similarly, the use of two non-linear equations to describe the shallow and deep data, for this cruise, would not improve the chlorophyll estimates much (since the r^2 accounts for >90% of the variance in the estimates) and would be complicated to apply in practice.

OC4 (Fig. 1c, 3c) provides an interesting case because there is little if any depth variability to the fluorescence, but there are large residuals at the higher chlorophyll values. This may because of the highly significant time variability seen on this cruise (Fig. 4d, Table 3). OC4 had few profiles and which were widely spaced in time. Obtaining enough data points produced variations in R and larger residuals.

The more interesting changes in R are with depth, shown for OC2 and OC3 (Fig. 4a,c) where it was found to be a highly significant source of variation (Table 3). There is near-surface variability in both, and OC3 shows a distinct minimum in the upper part of the broad chlorophyll maximum (see Fig. 1b). This secondary variability in R may contain useful information about photosynthesis, photoadaptation and, perhaps, species composition (see Cullen, 1982). However, the variability in R between cruises makes interpretation of that parameter difficult. Nevertheless, this deserves further study.

The at-sea and laboratory calibrations for OC4 show different offsets at zero chlorophyll a (Fig. 2d). Linear regressions on both laboratory and at-sea samples have regression coefficients which are not significantly different from one another ($P<0.05$). By adding the difference in the x-axis intercepts to the fluorescence values (filled triangles in Fig. 2d), much of the difference in the laboratory and field calibrations disappears. This means that the fluorometer was measuring a fluorescence missed by our filtration method. Therefore, the offset between the at-sea and laboratory calibrations on OC4 may be explained by particulate chlorophyll able to pass the GF/F filter, or else from a dissolved substance with similar fluorescence characteristics. Parker (1981) has found that fluorescence from dissolved organic matter is a small fraction of the particulate fluorescence, which suggests that the differences we see between laboratory and at-sea calibrations is more likely due to a filterable organism. Taguchi and Laws (1988) observed a population of microparticles which pass GF/F filters. Phinney and Yentsch (1985) observed a similar phenomenon. Although Taguchi and Laws' (1988) site differs from ours, the quantity of chlorophyll passing the filters (about 30%) and the seasonal distribution of these microparticles (maximum in fall or winter) are similar. Chisholm et al. (1988) have also documented organisms containing a chlorophyll that may be sensitive to the fluorescence excitation, however, these should have been retained by the GF/F filter (Chisholm et al., 1988). If this fluorescence is from filterable particles at this time of year, they have similar R values as on the other cruises, as indicated by the regression coefficients in Table 2

In conclusion, these data justify the use of in situ fluorometers for the estimation of chlorophyll a at sea; in vivo fluorescence is more than an 'indicator' of chlorophyll variability. Although we see evidence implicating photoadaptation and population shifts, these are minor and not consistent and do not compromise the estimate of

chlorophyll in the environments we sampled. The agreement between laboratory and field calibrations means that laboratory calibrations can be used to estimate chlorophyll changes from sensors placed on moorings. Furthermore, for typical oceanic values of chlorophyll, these laboratory calibrations are stable. As long as there is biological variability, the fluorescence calibration in terms of chlorophyll will always be inexact. However, much of the variability appears to be secondary.

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Fig. 1. Plots of temperature (T), fluorescence (fluor),and beam attenuation coefficient (b.a.c.) for three of the Biowatt II cruises (OC2, OC3, OC4) and the ML-ML cruise (E1). OC2, OC3 and OC4 were to the North Sargasso Sea (34°N/70°W), and E1 was to the Gulf of Maine (43°N/69°W). For the OC cruises, salinity is invariant over depth, except for a slight (i.e., <0.3 psu) freshening in summer, thus temperature is an adequate estimator for the density changes with depth. For E1, the large temperature range also makes it the largest contributor to density.

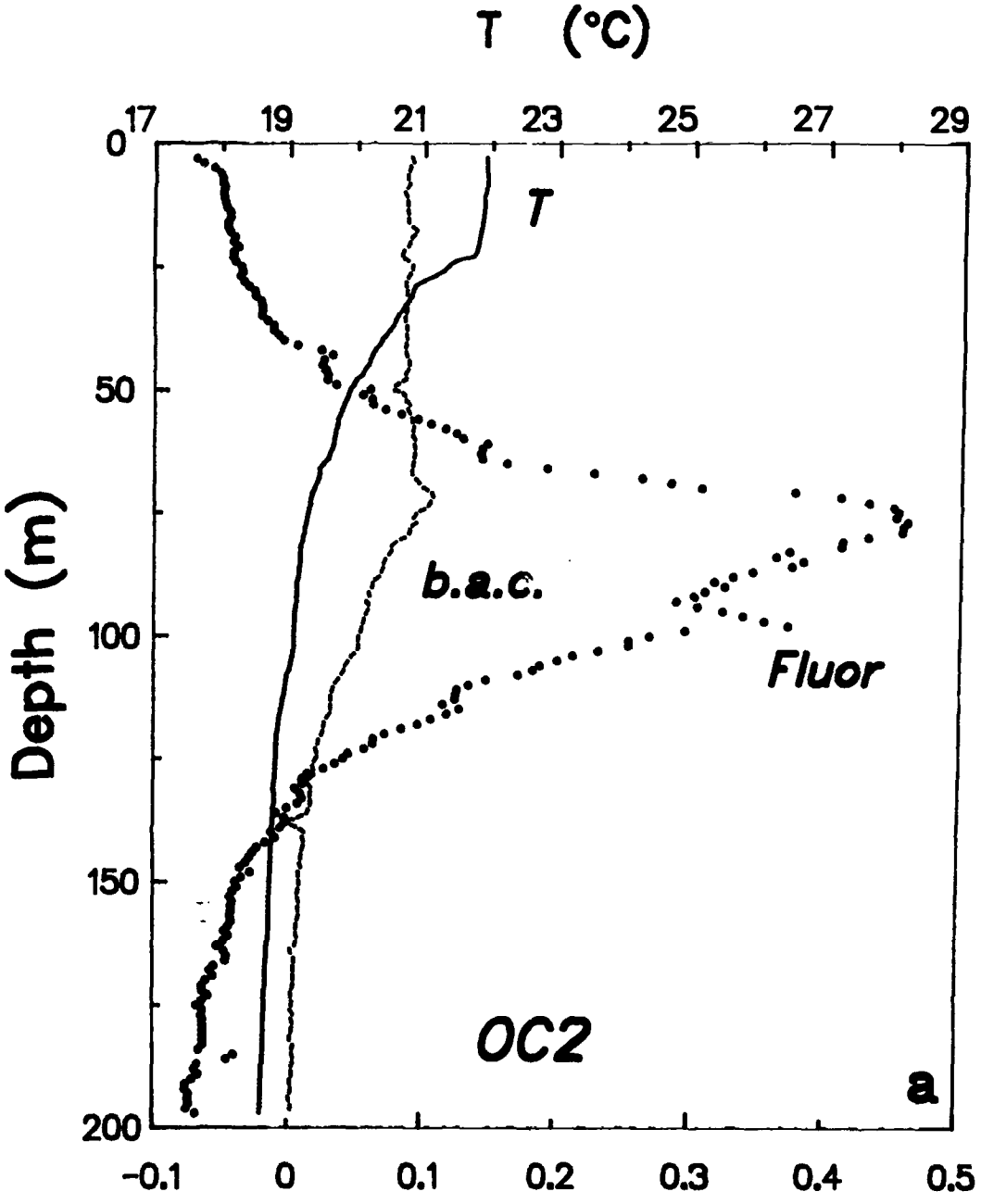
T (°C)
17
19
21
23
25
27
29
0
50
100
150
200
Depth (m)
T
b.a.c.
Fluor
OC2
a
-0.1
0
0.1
0.2
0.3
0.4
0.5

Fig 1a
Marra

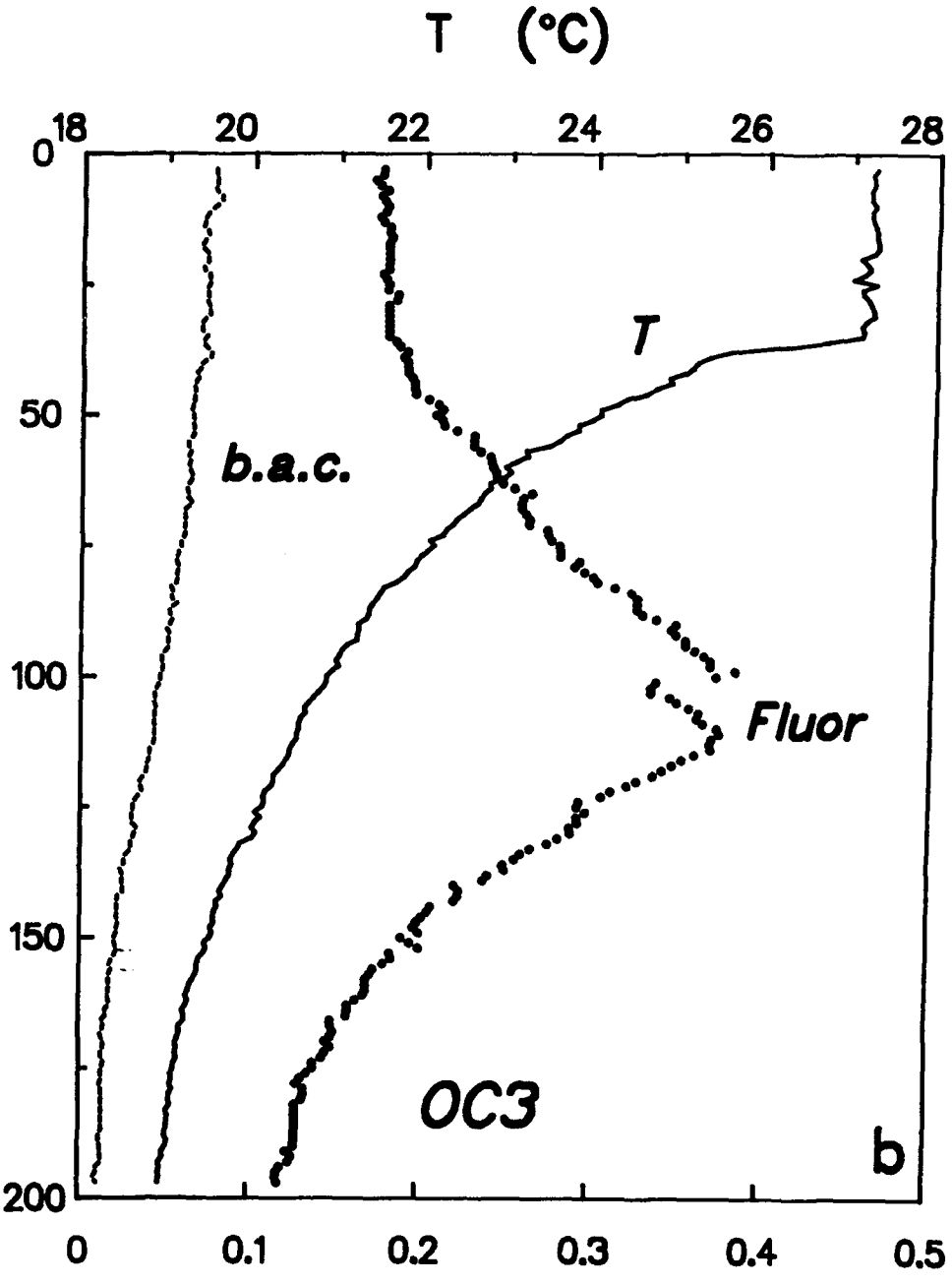
T (°C)
18
20
22
24
26
28
0
50
100
150
200
T
b.a.c.
Fluor
OC3
b
0
0.1
0.2
0.3
0.4
0.5

Fig. 1b

Marra

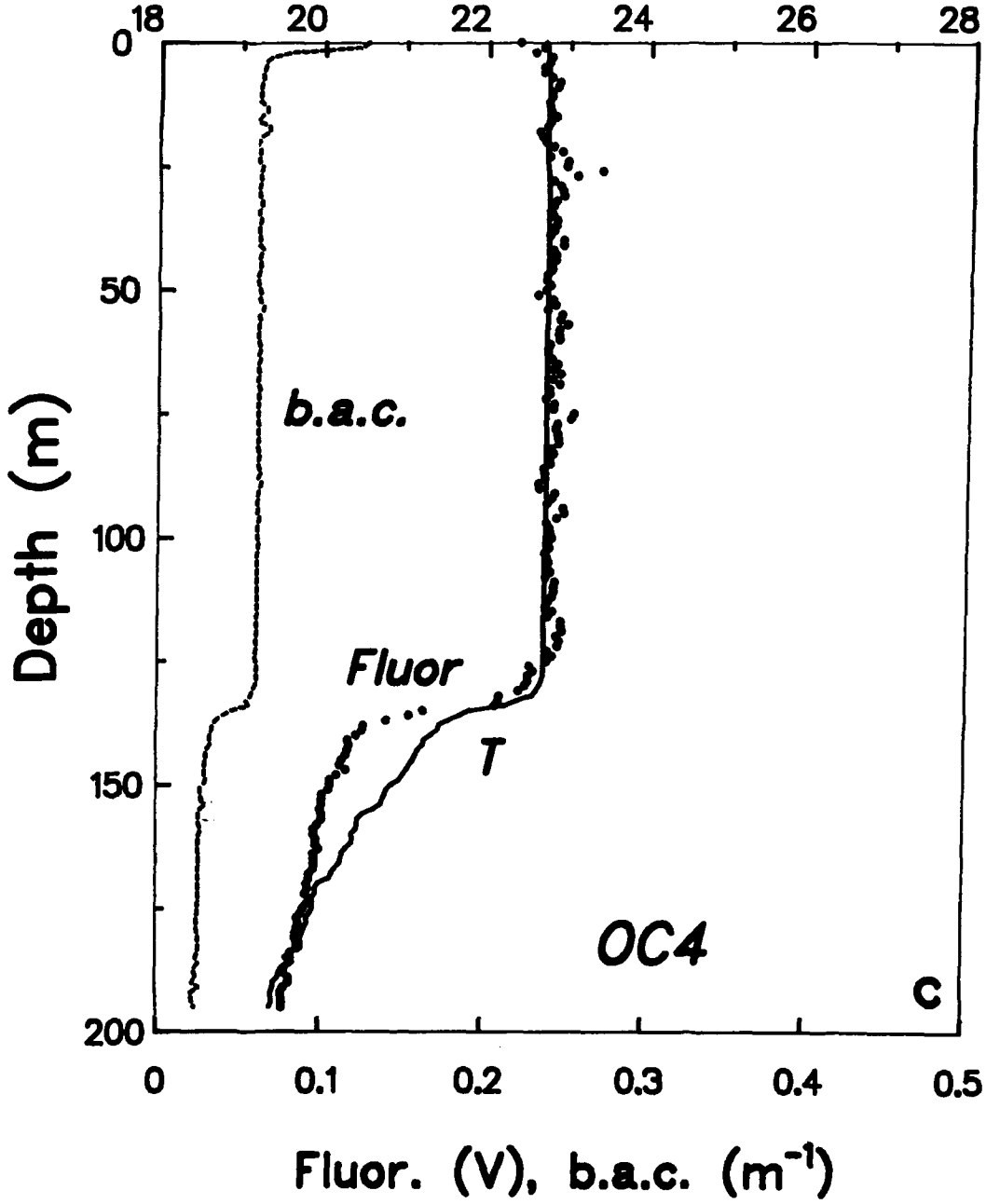
18
20
22
24
26
28
0
50
100
150
200
Depth (m)
b.a.c.
Fluor
T
OC4
C
0
0.1
0.2
0.3
0.4
0.5
Fluor. (V), b.a.c. (m⁻¹)

Fig. 1c

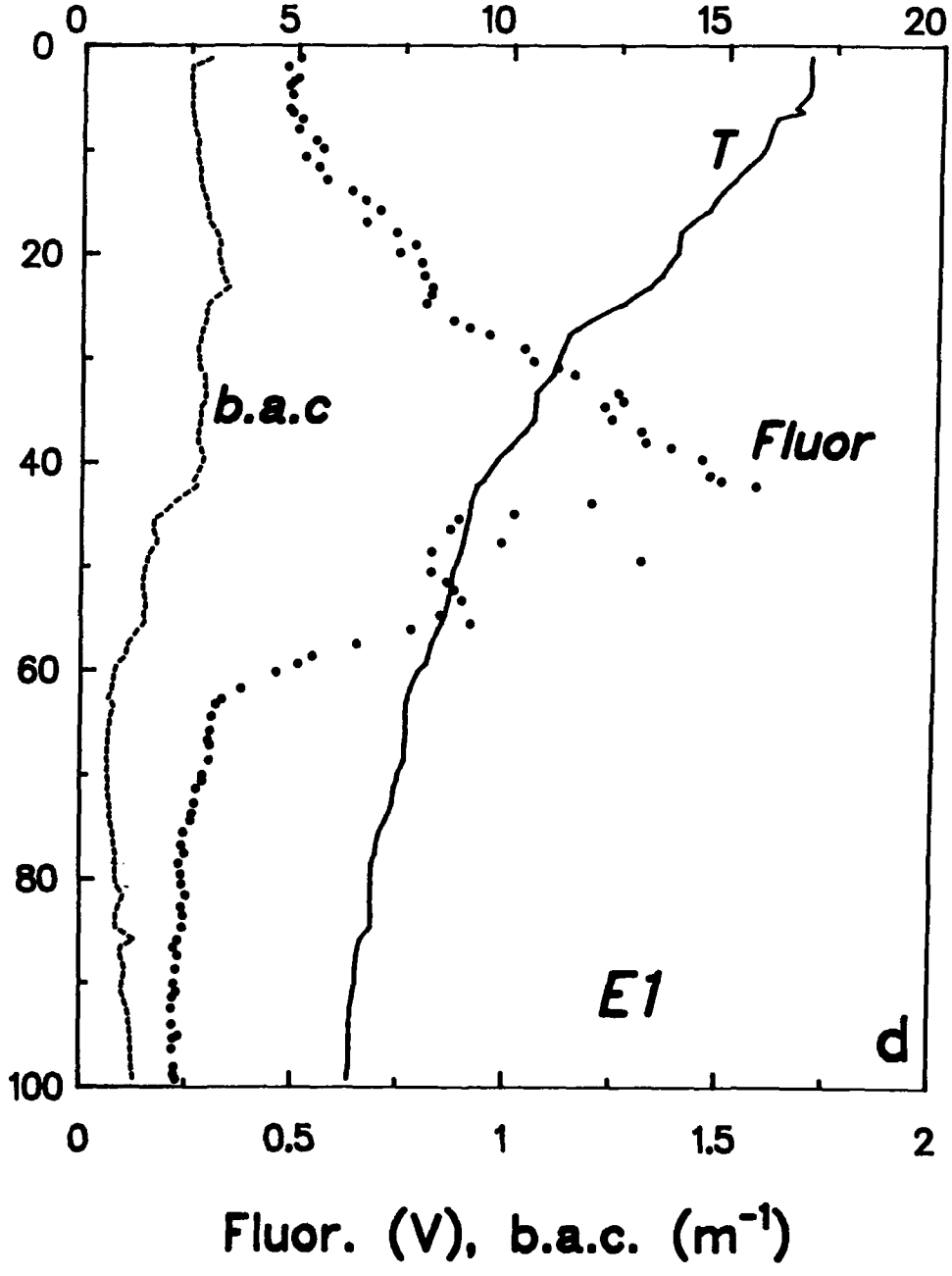
0
5
10
15
20
0
20
40
60
80
100
T
b.a.c
Fluor
E1
d
0
0.5
1
1.5
2
Fluor. (V), b.a.c. (m⁻¹)

Fig 1d.

Fig. 2. Calibrations from the four cruises (OC2, OC3, OC4, E1). At-sea samples are shown as dots, and laboratory data are shown as triangles. The regression lines (see Table 2) are for the at-sea data. For OC4, we have "corrected" the laboratory calibration data (filled symbols) for illustration purposes, as described in the text.

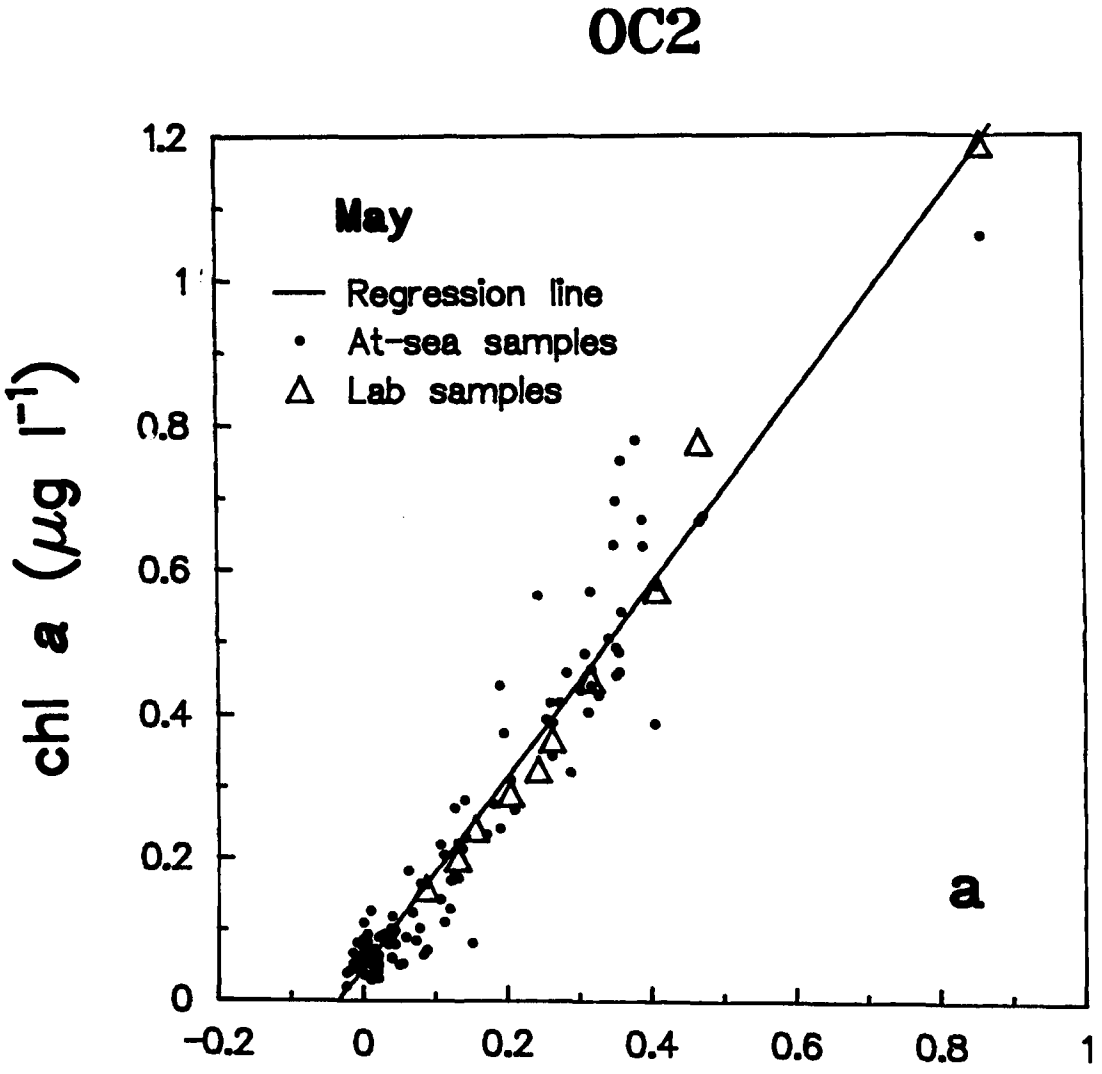
OC2
May
Regression line
At-sea samples
Lab samples
chl a (μg l⁻¹)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.2
0.4
0.6
0.8
1
a

Fig 2a
Marra

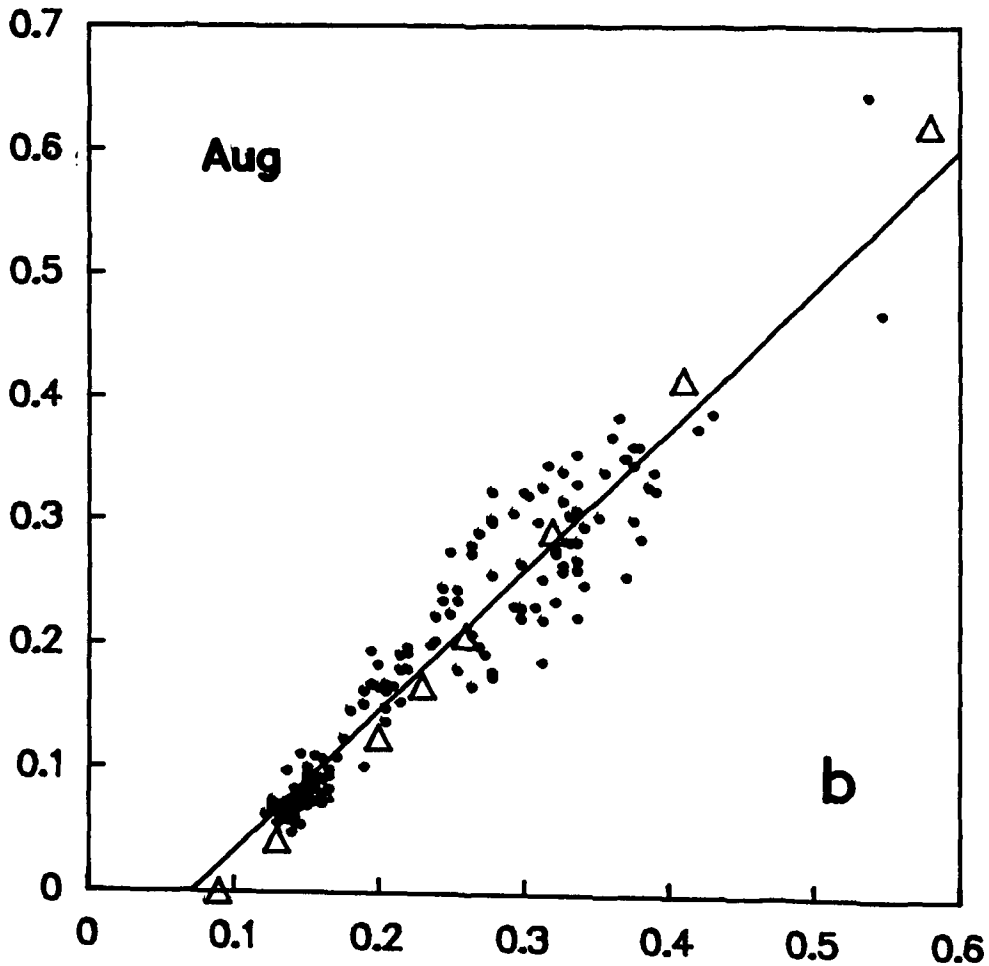

Fig 2b
Mann

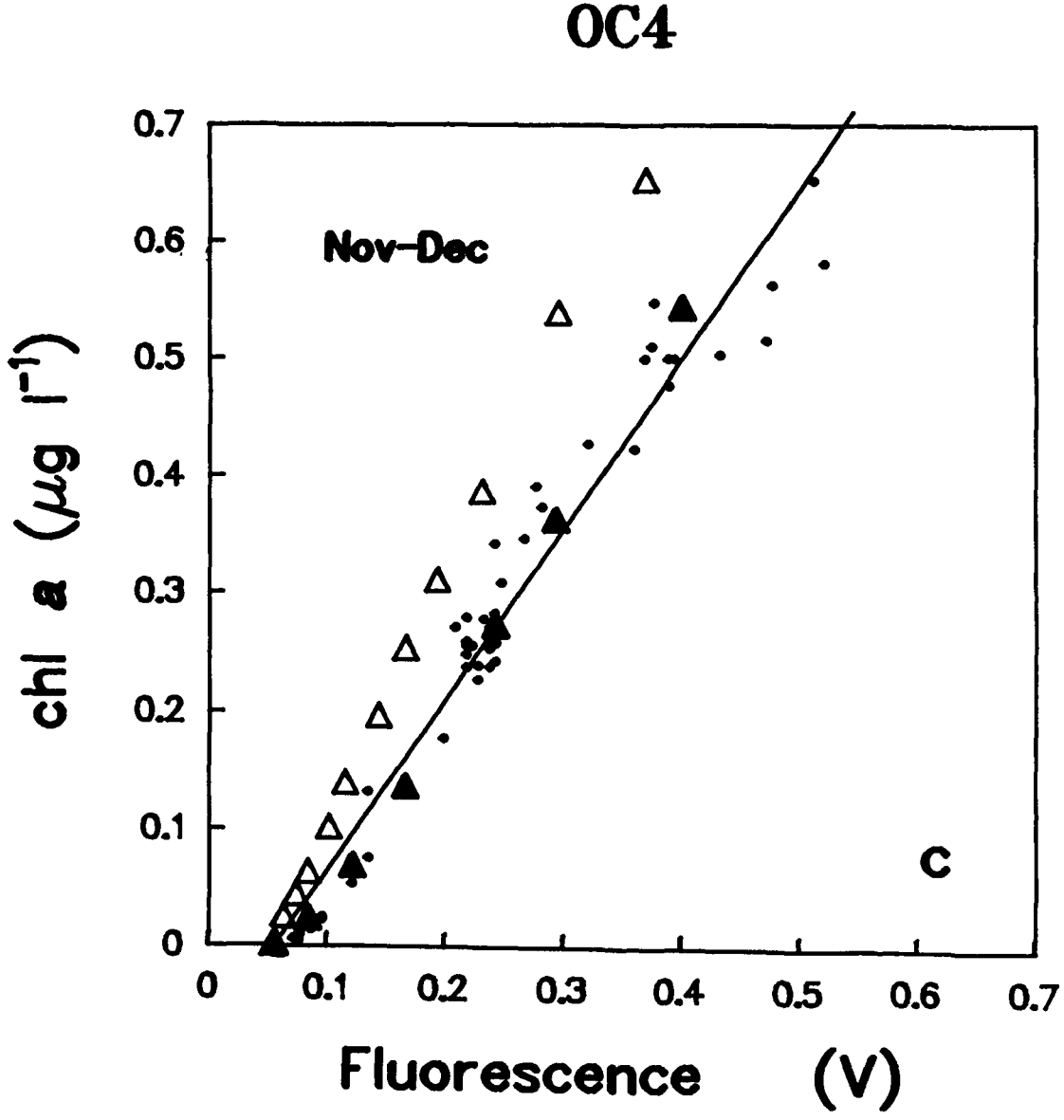

Fig 2c
Mar

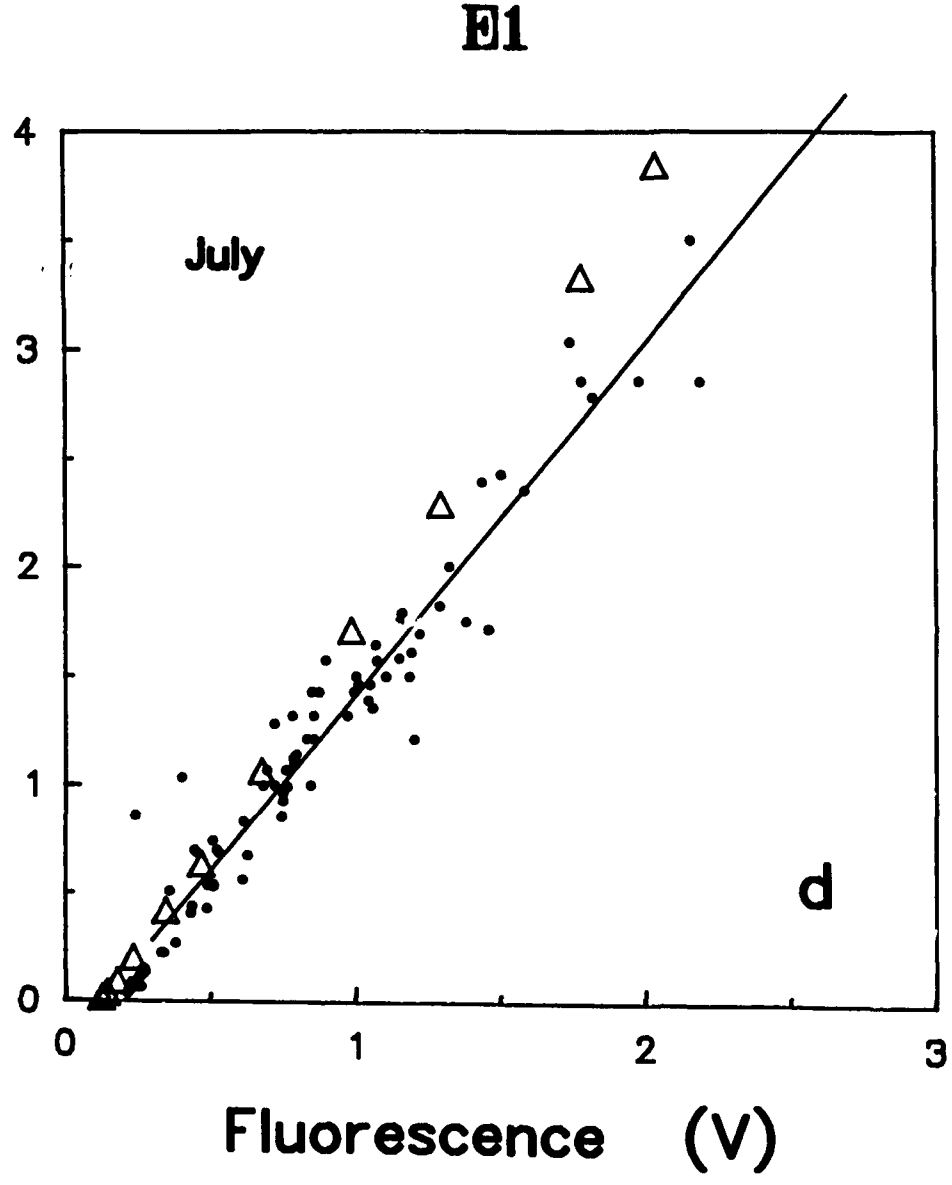

Fig 2d

Fig. 3. Standardized residuals (equation (1) in text) from the four cruises plotted against chlorophyll a from the regression.

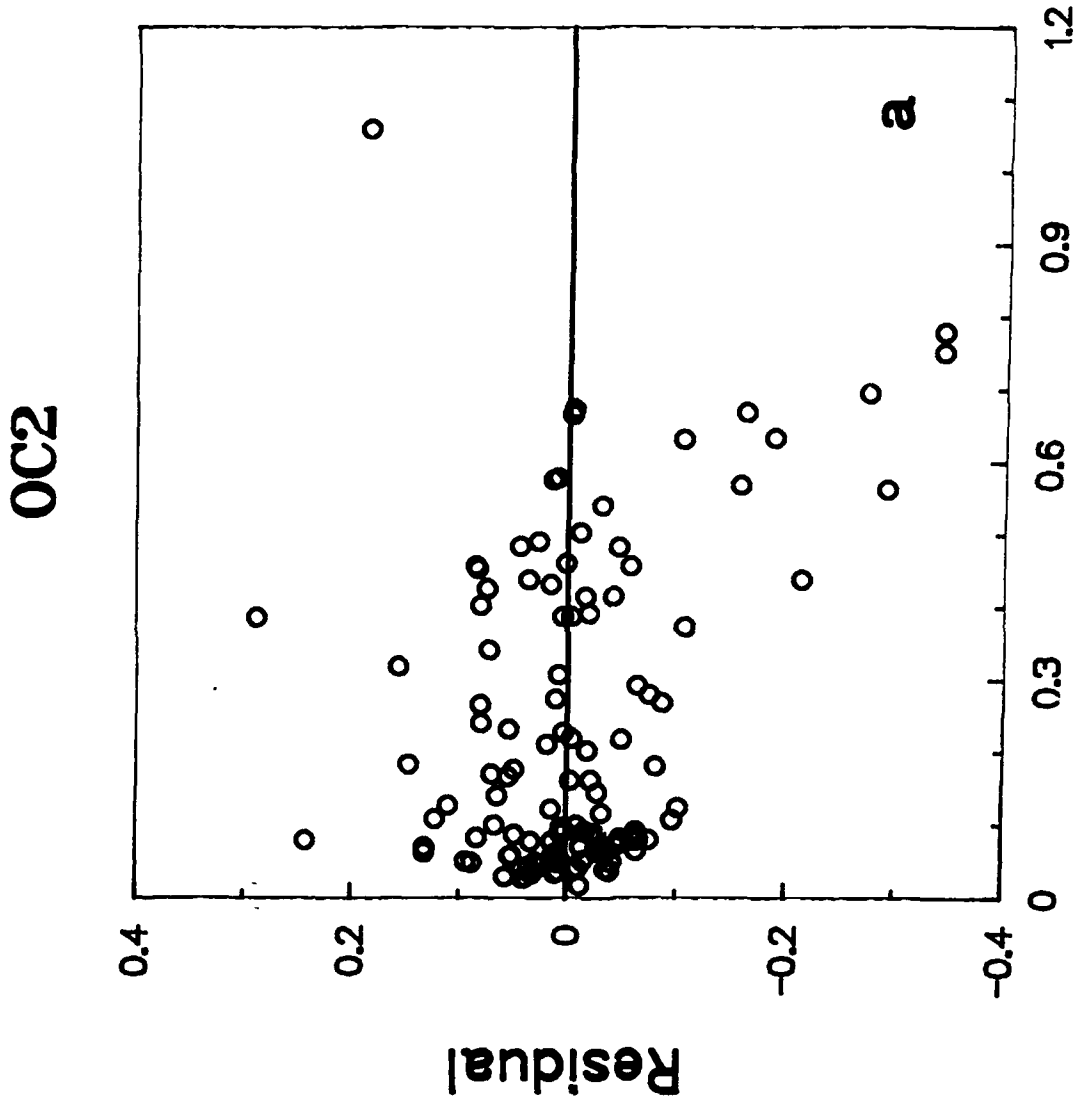

Fig. 3a

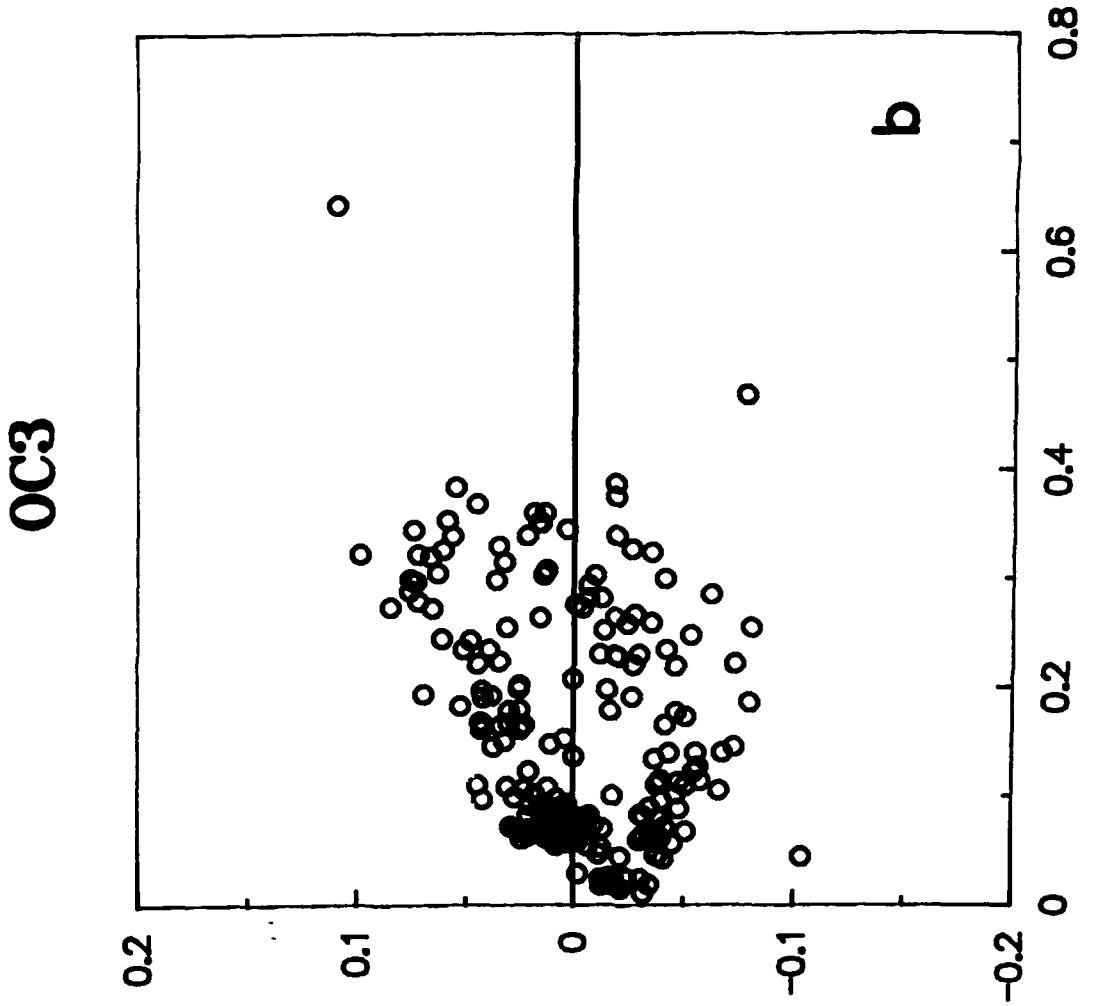

Fig 3b
Marra

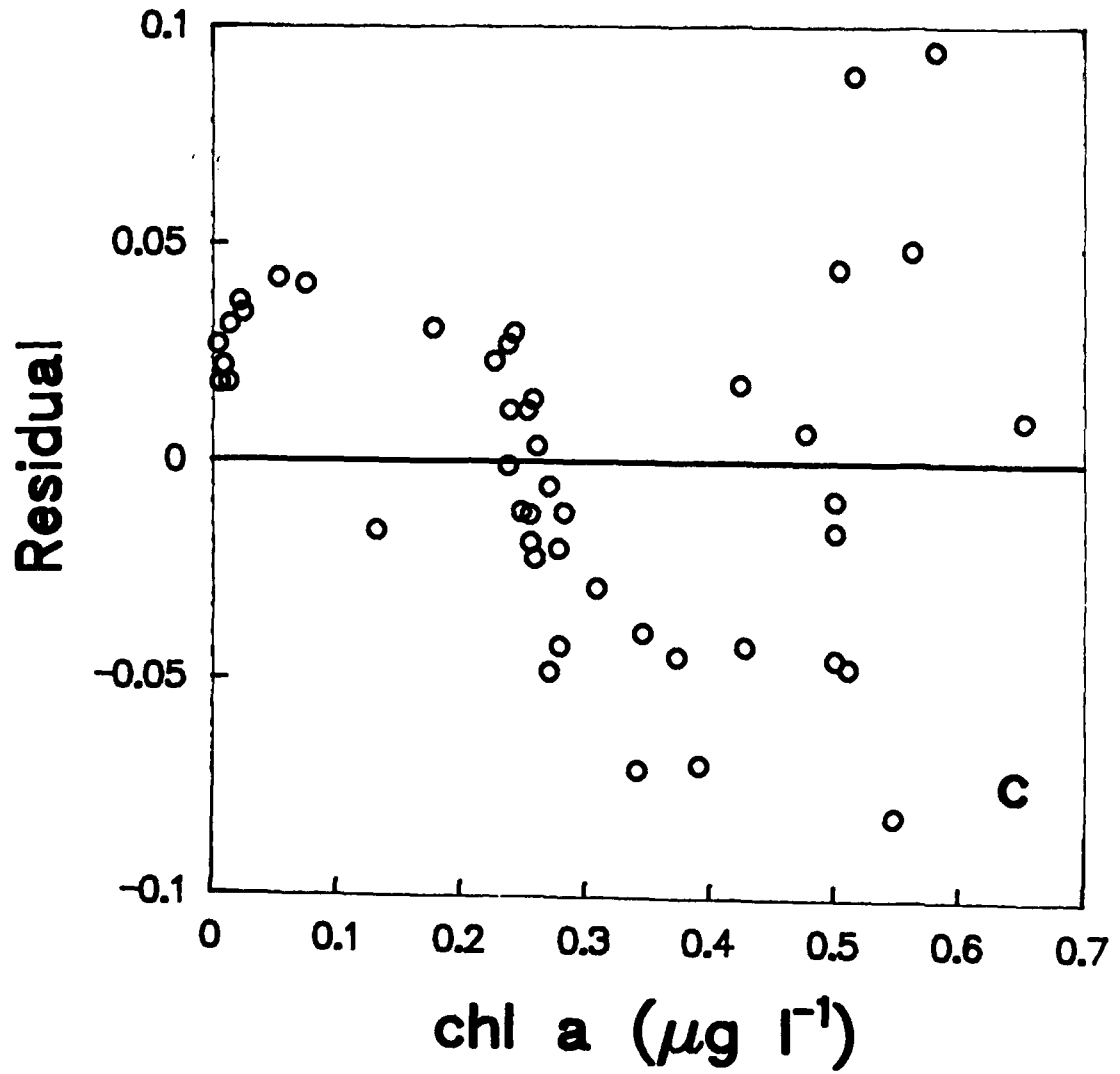

Fig 3c
Mana

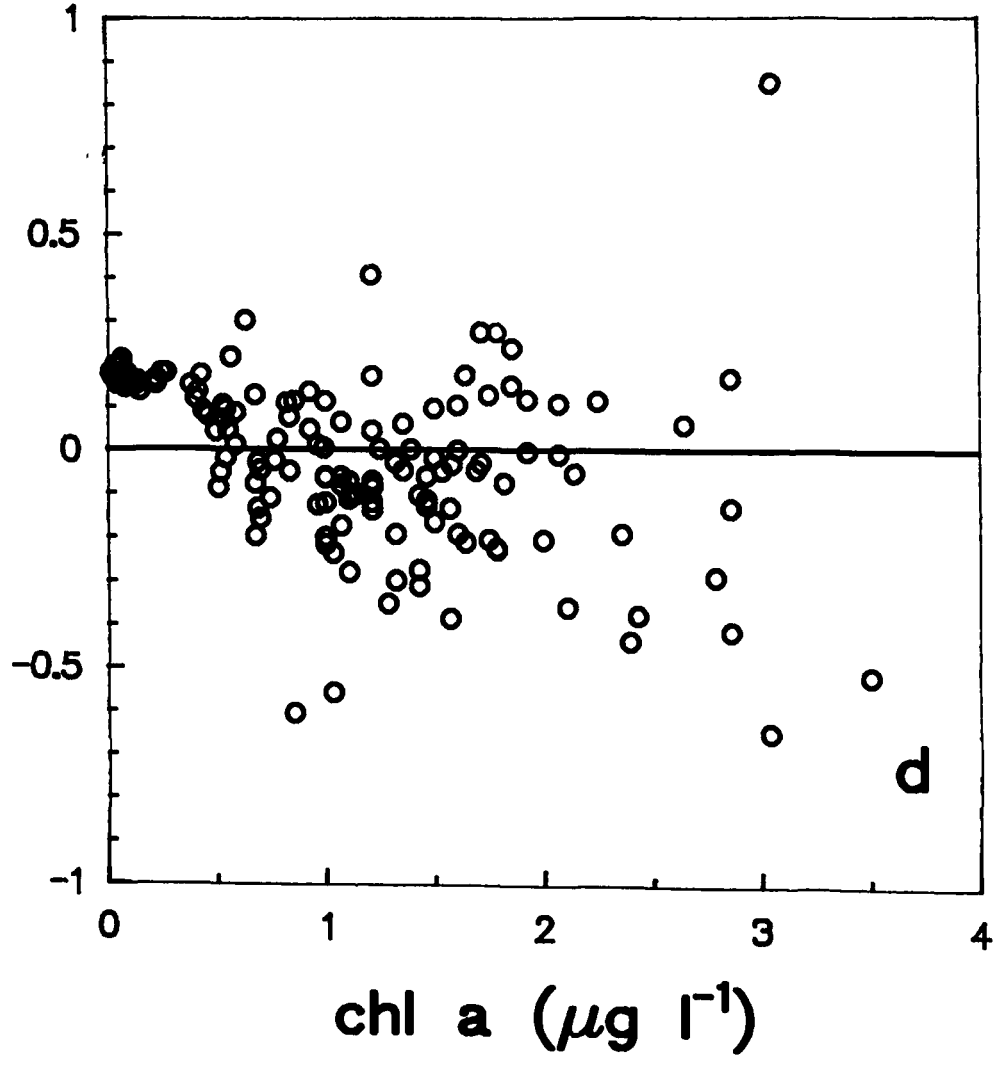

Fig 3d
Mann

Fig. 4. Plots of R against sources of variation found to be highly significant from the multiple regression analysis (see Table 3).

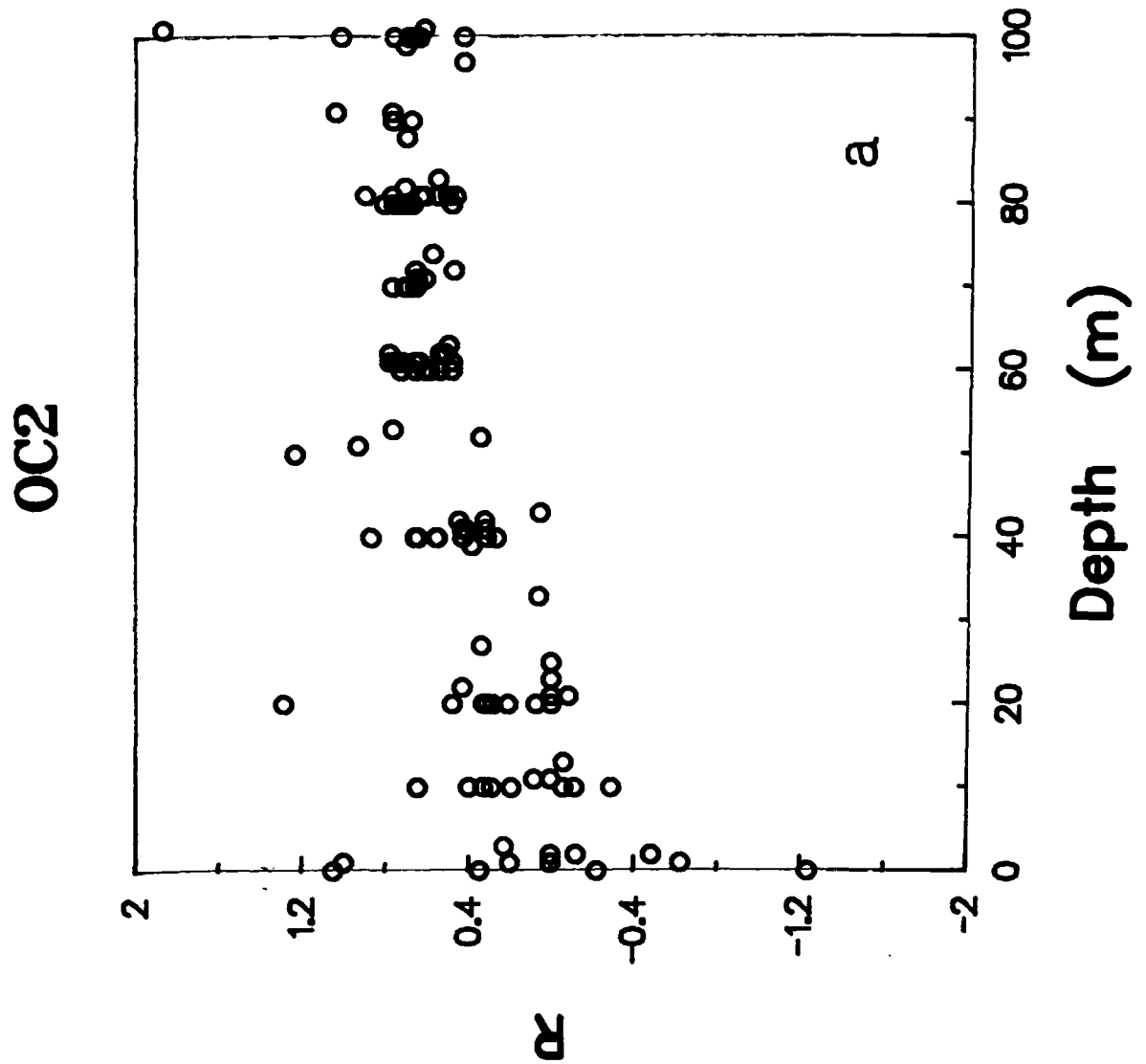

Fig 4a
Mara

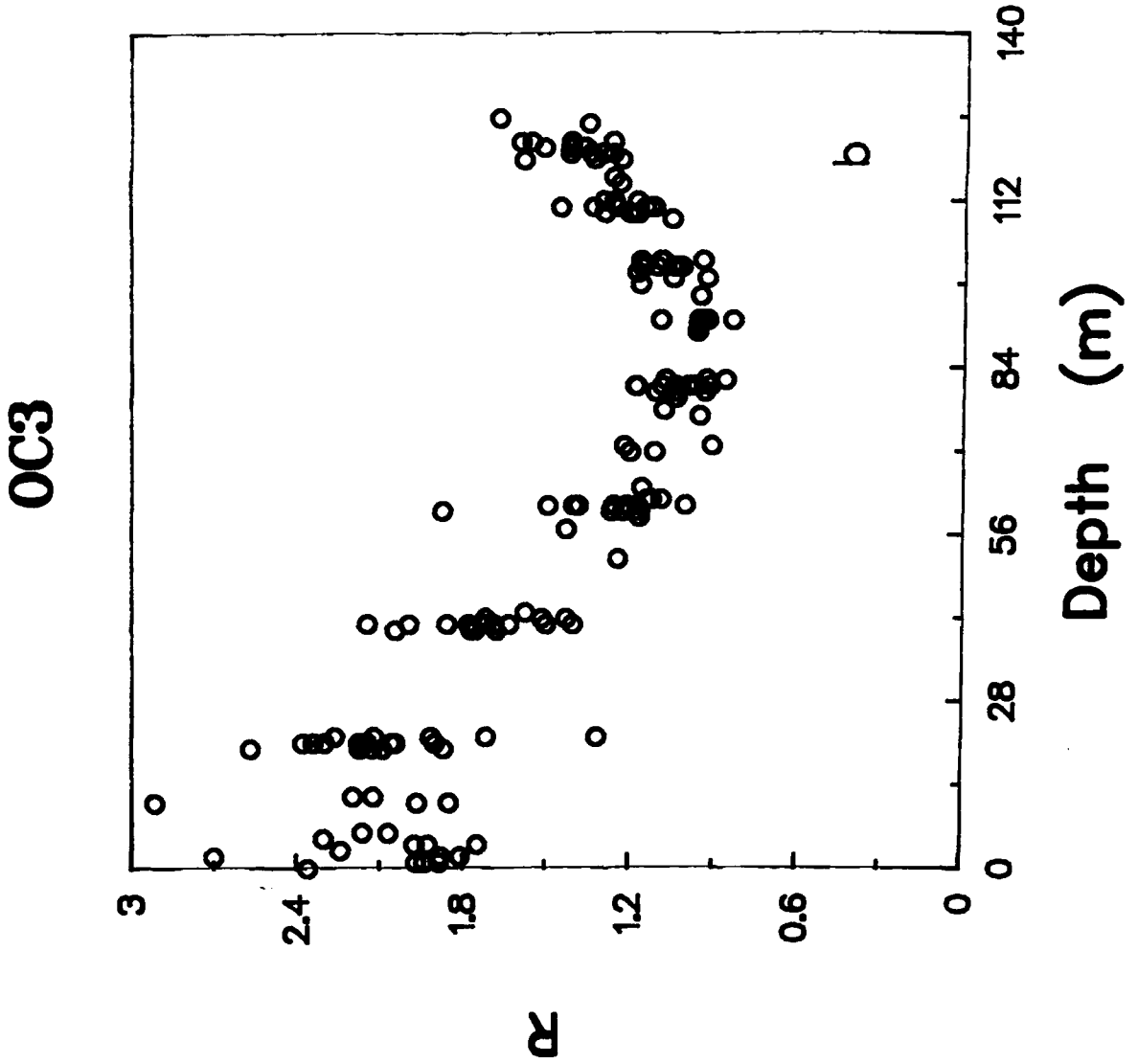

Fig 45
Marra

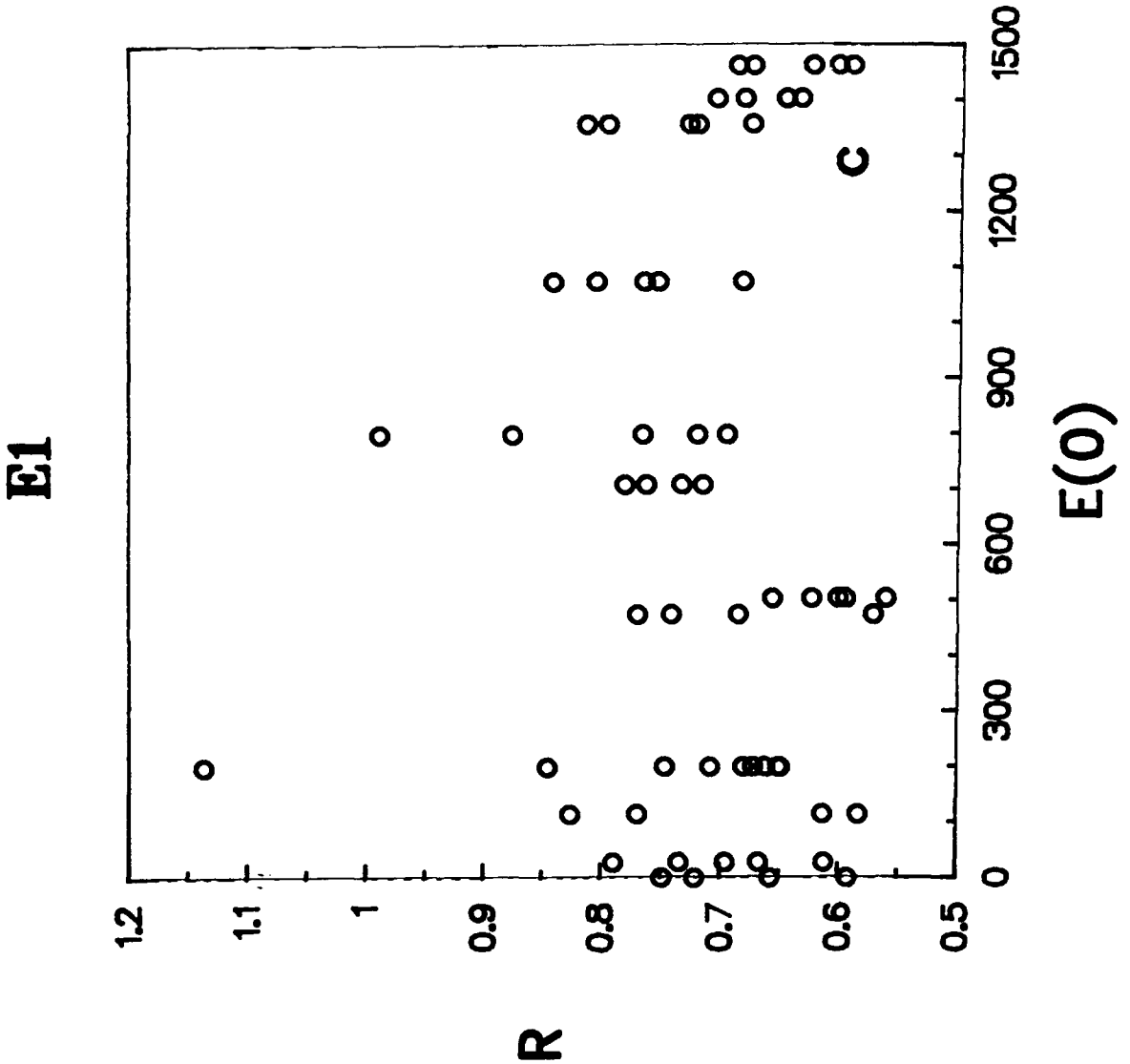

Fig 4c
Mann.

Fig. 5 An example of a series of laboratory calibrations of two fluorometers. Calibration dates are given in the legends.

Ser. No. 07

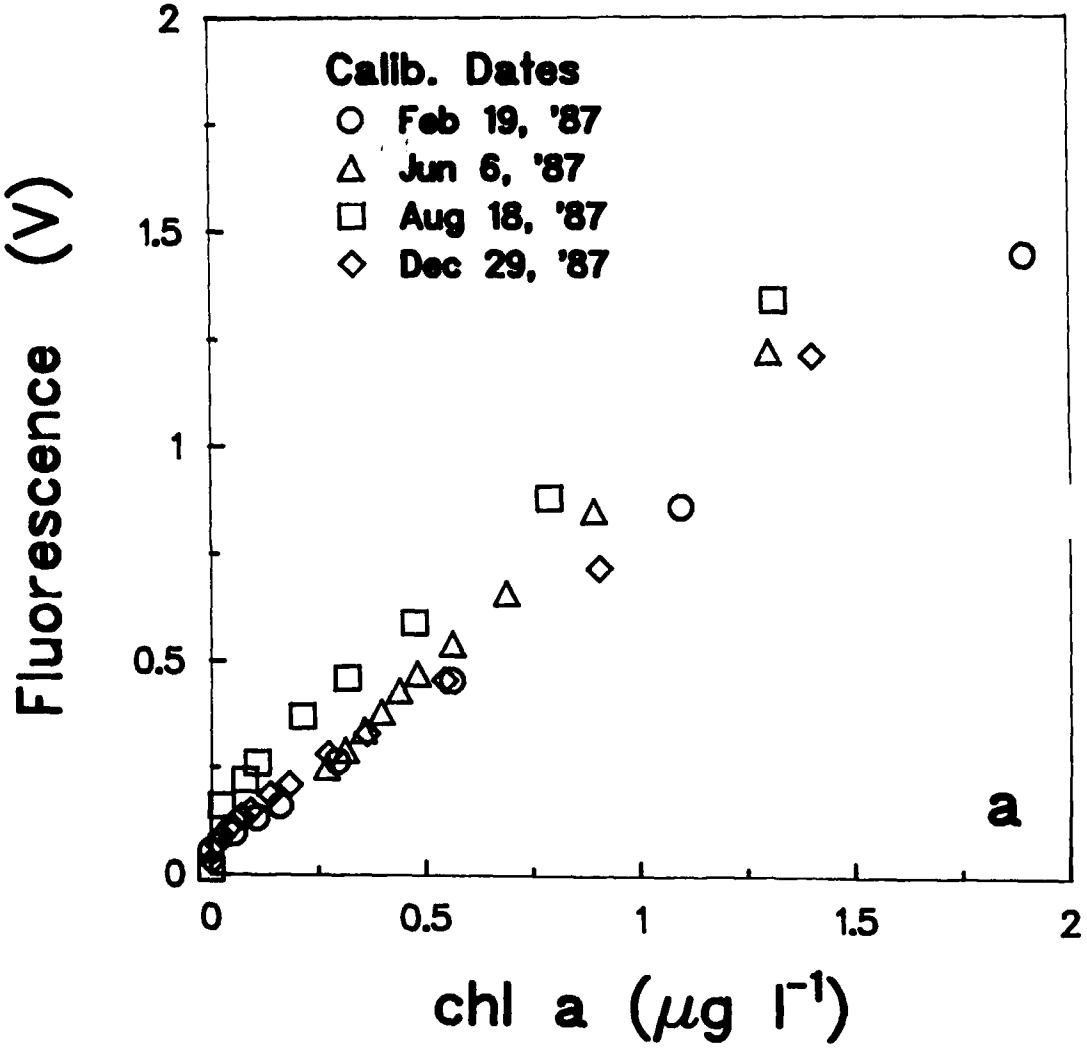

Fig 5a
Marra

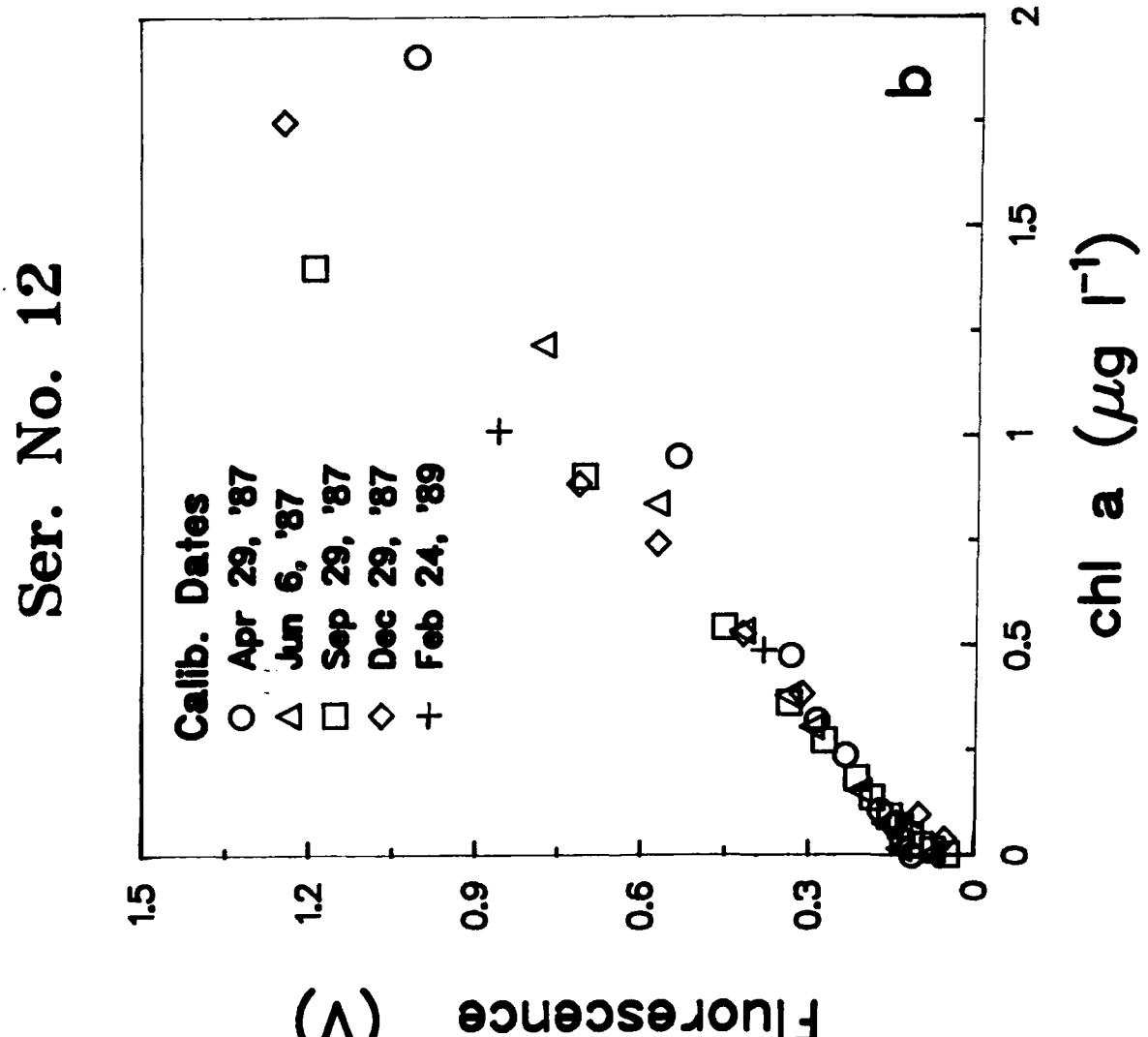
Ser. No. 12
Calib. Dates
Apr 29, '87
Jun 6, '87
Sep 29, '87
Dec 29, '87
Feb 24, '89
b
Fluorescence (V)
chl a (μg l⁻¹)
1.5
1.2
0.9
0.6
0.3
0
0
0.5
1
1.5
2

Fig. 5b
Marra

Table 1. Cruise description and euphotic depths. Dates listed are periods over which data was collected. The mean euphotic depth (for PAR) for each cruise is given as the 1% light depth.

Cruise	Dates	1% E depth (m)
OC2	9 - 21 May,'87	100
OC3	22 - 25 Aug.,'87	125
OC4	20 Nov. - 1 Dec.,'87	80
E1	23 July - 1 Aug.,'90	25

Table 2. Regression equations of the relationship between in situ fluorescence (F) and chlorophyll a (chl), along with the standard error of the regression coefficient in the estimate of chl and the coefficient of determination (r2).

Cruise	Regression Equation	SE	r^2
OC2	chl = 1.33F+0.047	± -0.028	0.92
OC3	chl = 1.15F-0.083	0.030	0.90
OC4	chl = 1.45F-0.270	0.046	0.92
E1	chl = 1.43F-0.092	0.037	0.91

Table 3. A multiple regression analysis of day of sample (within cruise), the time of day (hour) at which the sample was taken and the depth of the sample (m) against R . The 'F-statistic' is given as a measure of the significance of the source of variation, as well as the probability, P, that the the source of variation is caused by random variation alone.

Cruise	Source of Variation	F	P	
OC2	Day	4.32	0.04	*
	Time	13.35	0.00	***
	Depth	87.41	0.00	***
OC3	Day	1.20	0.27	ns
	Time	2.06	0.15	ns
	Depth	100.00	0.00	***
OC4	Day	126.76	0.00	***
	Time	2.68 0.11	ns	
	Depth	1.78 0.19	ns	
E1	Day	1.61	0.19	ns
	Time	3.99	0.05	*
	Depth	0.14	0.71	ns

ns $P > 0.05$
* $0.01 < P < 0.05$
** $0.001 < P < 0.01$
*** $P < 0.001$

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In situ fluorometers are evaluated in their estimation of chlorophyll a. Calibrations from at-sea and laboratory data showed linear relationships between fluorescence and chlorophyll a, as measured by in situ fluorometers with $r^2 > 0.9$. Examination of regression residuals showed an increasing error variance with the magnitude of chlorophyll on two of four cruises. The most likely source of this increasing error variance was in one case, a photoadaptation effect and in the other a population

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shift between the beginning and end of the cruise. S. aller variability was also found in the ratio fluorescence to chlorophyll a, traced to sample depth, and time of day, although this variability was not a consistent property of the data. Generally, there was excellent agreement between laboratory and at-sea calibrations for low levels of chlorophyll typical of oceanic environments. The laboratory calibration of these instruments was stable over time, suggesting that good estimates of chlorophyll a can be made from fluorometers placed on ocean moorings.

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